IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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For : Cytokine production-inducing antibody

DECLARATION

I, Nicolas Torno, c/o Cabinet Regimbeau, 20 rue de Chazelles, 75017 Paris (France), hereby declare that I am well acquainted with the French and English languages and hereby certify that to the best of my knowledge and belief the following is a true translation of French priority n° 02 11416 filed on September 13, 2002.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

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The present invention relates to a method for measuring the activation of an effector cell belonging to the immune system, or modified in vitro, by means of a monoclonal (MoAb) or polyclonal antibody, characterized in that it comprises bringing CD16 receptor-expressing cells into contact in a reaction medium in the presence of the antibody and of the antigen for said antibody, and measuring the amount of at least one cytokine produced by the CD16 receptor-expressing cell.

Immunotherapy by means of polyclonal or monoclonal antibodies is in the process of becoming one of the most important aspects of medicine. On the other hand, the results obtained in clinical trials appear to be contrasting. In fact, the monoclonal antibody may prove to be insufficiently effective. Today, research is directed toward the immunoglobulin Fcγ fragment in order to improve antibody properties. In the end, this should make it possible to obtain antibodies which interact with and activate the receptors of effector cells (macrophage, T lymphocyte and NK cell).

The biological activity of certain immunoglobulins G is dependent on the structure of the oligosaccharides present on the molecule, and in particular on its Fc portion. The IgG molecules of all human and murine subclasses have an N-oligosaccharide attached to the CH_2 domain of each heavy chain (at residue Asn 297 for human IgGs). The influence of this glycan residue on the ability of the antibody to interact with effector molecules (Fc receptors and complement) has been demonstrated. Inhibiting glycosylation of a human IgG1, by culture in the presence of tunicamycin, results for example in a 50-fold decrease in the affinity of this

antibody for the FcγR1 receptor present on monocytes and macrophages (Leatherbarrow et al., 1985). Binding to the FcγRIII receptor is also affected by the loss of carbohydrates on IgG, since it has been described that an unglycosylated IgG3 is incapable of inducing ADCC-type lysis by the FcγRIII receptor on NK cells (Lund et al., 1990).

However, beyond the necessary presence of these glycan residues, it is more precisely the heterogeneity of their structure which can result in differences in the ability to engage effector functions. Galactosylation profiles that are variable according to individuals (human serum IgG1s) have been observed. These differences probably reflect disparities between the activity of galactosyltransferases and other enzymes between the cellular clones of these individuals (Jefferis et al., 1990). Although this normal heterogeneity of post-translational processes generates various glycoforms (even in the case of monoclonal antibodies), it can result in atypical structures associated with certain pathological states such as rheumatoid arthritis or Crohn's disease, for which a considerable proportion of agalactosylated residues has been demonstrated (Parekh et al., 1985).

Faced with the complexity posed by the relationship that exists between the various glycan structures and the activity of antibodies, it would be useful to be able to rapidly discriminate which antibodies are effective, and thus make it possible to select cell lines producing antibodies having greater effectiveness or specific properties in the activation or inhibition of certain components of the immune system.

In application FR 0004685 of April 12, 2000 (LFB), we have described a novel method for preparing a monoclonal antibody capable of activating effector cells expressing FcγRIII. In this method, monoclonal

antibodies originating from hybridomas or from transfected lines are tested in a reaction mixture comprising the target cells of said antibodies, effector cells comprising FcγRIII-expressing cells, and polyvalent IgGs. Thus, it is possible to determine the percentage lysis of the target cells and to select monoclonal antibodies which activate the effector cells, causing significant lysis of the target cells (FcγRIII-type ADCC activity). For example, the Fab portion of the anti-D antibody will bind to the Rhesus D antigen carried by red blood cells. Subsequent to this binding, its Fc portion then binds to the Fc gamma RIII receptor, or CD16, of the effector cell (NK cell). This "sandwich" induces the secretion of chemical substances such as perforins which will lyse the red blood cell. This is therefore an antibody-dependent cellular cytotoxicity (ADCC). In order to be close to physiological conditions, the test is carried out in the presence of human polyvalent immunoglobulins.

In the context of the invention, it has been found that the binding of an antibody to its ligand can induce activation of CD16-transfected Jurkat cells, inducing IL2 secretion. A strong correlation is observed between the secretion of IL2 by Jurkat CD16 and the CD16-mediated ADCC activity of the effector cells.

The invention therefore proposes the use of antibodies selected using a Jurkat CD16 test, by measuring secreted IL2 as an alternative to ADCC tests, in particular to follow and screen the biological activity of antibodies for therapeutic use.

Description

Thus, the present invention relates to a method for measuring the activation of an effector cell belonging to the immune system, which may or may not be transformed, by means of a monoclonal (MoAb) or

polyclonal antibody characterized in that it comprises bringing CD16 receptor-expressing cells into contact in a reaction medium in the presence of the antibody and of the antigen for said antibody, and measuring the amount of at least one cytokine produced by the CD16 receptor-expressing cell.

The term "transformed cell" is intended to mean a cell that has been genetically modified so as to express a receptor, in particular the CD16 receptor.

Preferably, a Jurkat line transfected with an expression vector encoding the CD16 receptor is used as effector cell.

Among the cytokines that may be quantified, it is possible to measure the production of at least one cytokine selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, ..., TNFα and TGFβ. The interleukin IL-2 may advantageously be chosen. The amount of cytokine produced is a marker for activation or for inhibition of effector cells.

Preferably, the amount of interleukin IL2 secreted reflects the quality of the antibody bound by the CD16 receptor as regards its antigen-binding integrity (Fc function) and effectiveness (antigenic site). The measurement of the amount of IL2 is correlated with an ADCC-type activity.

In another aspect, the invention relates to a method for evaluating the effectiveness of a monoclonal or polyclonal antibody, characterized in that it comprises bringing CD16 receptor-expressing effector cells of the immune system, which may or may not be transformed, into contact in a reaction medium in the presence of an antibody and of the antigen for said antibody, and measuring the amount of at least one cytokine produced by the CD16 receptor-expressing cell.

This method is particularly suitable for evaluating the effectiveness of a monoclonal or polyclonal antibody having an anti-human red blood cell Rh D specificity.

In another aspect, the invention relates to a method for evaluating the ability of a cell to produce an effective monoclonal antibody, characterized in that it comprises bringing CD16 receptor-expressing effector cells of the immune system, which may or may not be transformed, into contact in a reaction medium in the presence of an antibody and of the antigen for said antibody, and measuring the amount of at least one cytokine produced by the CD16 receptor-expressing cell.

This method can be implemented for cells used for the production of therapeutic antibodies, such as CHO, YB2/0, human lymphoblastoid cells, insect cells and murine myeloma cells.

This method may also be applied to the evaluation of MoAb production by transgenic plants or transgenic mammals.

In a complementary aspect, the invention is directed toward a method for evaluating the effectiveness and the integrity of polyclonal antibodies after one or more purification steps, characterized in that it comprises bringing CD16 receptor-expressing effector cells of the immune system, which may or may not be transformed, into contact in a reaction medium in the presence of the purified antibody and of the antigen for said antibody, and measuring the amount of at least one cytokine produced by the CD16 receptor-expressing cell.

The methods described above can optionally be carried out in the presence of human immunoglobulins (IVIgs).

By way of example, antibodies for which an increase of more than 100%, 250%, 500% or 1000% in the amount of IL-2 release is observed compared with the control in the absence of antibody, or a given antibody as negative reference, will be selected.

The invention is also directed toward the use of the method described above, for selecting antibodies that are effective for a therapeutic treatment. Alternatively, the invention is also to evaluate the the response ability of a patient effector cells when they are in presence of a polyclonal or monoclonal antibody for treating the patient and when there are in the conditions according to the invention.

Legends

Figure 1: Description of the MNC ADCC assay

Mononuclear cells in the presence of Tegeline (IVIg) are incubated with the anti-Rhesus D antibodies and Rhesus + red blood cells (target). After an overnight period at 37°C, the red blood cell lysis is measured by evaluating the amount of hemoglobin released into the reaction medium.

Figure 2: Description of the NK ADCC assay

Purified NK cells are incubated with the anti-Rhesus D antibodies and Rhesus + red blood cells (target). After an overnight period at 37°C, the red blood cell lysis is measured by evaluating the amount of hemoglobin released into the reaction medium.

Figure 3: NK ADCC results and inhibition with the anti-CD16 "3G8"

Figure 4: Description of the Jurkat CD16 assay

Jurkat CD16 cells are mixed with various anti-D antibodies in the presence of Rhesus + red blood cells and of PMA. After an overnight incubation period, the release of IL-2 into the supernatant is quantified by ELISA.

Figure 5: Results of the Jurkat CD16 assay

Comments: the antibodies that are positive in ADCC-NK induce secretion of IL2 in the presence of Jurkat CD16 and of their target.

Example 1: Jurkat CD16 assay

Antibodies:

WinRho polyclonal antibodies, DF5-EBV monoclonal antibody, DF5-YB2/0 monoclonal antibody.

Principle:

This assay evaluates the ability of the anti-D antibodies to bind to the CD16 receptor (Fc gamma RIII) expressed on Jurkat CD16 cells, and to induce IL2 secretion.
This assay consists in bringing the following into contact in a 96-well plate: the anti-D antibodies, the papain-treated Rhesus-positive red blood cells, the Jurkat CD16 cells and PMA.
After an overnight incubation period at 37°C, the 96-well plates are centrifuged and the amount of secreted IL2 is assayed in the supernatant.
Material
Positive control antibodies: Poly-D WinRho, DF5-YB2/0.
Negative control antibody: DF5.
Rhesus-positive red blood cells.
Jurkat CD16 cells.
IL2 assay kit: Quantikine from R/D.

Method

Treatment of red blood cells with papain.

1 ml of pellet of red blood cells diluted in PBS is incubated with 1 ml of a papain solution (1 mg/ml) for 10 min at 37°C. Three washes are then carried out in H_2O-0.15M NaCl.

Reaction mixture:

-Antibody: 50 µl of a dilution to 150 ng/ml in IMDM 5% SVF,

-PMA: 50 µl of a dilution to 40 ng/ml in IMDM 5% SVF,

-red blood cells treated with papain. 50 µl at 8×10^6/ml in IMDM 5% SVF,

-Jurkat CD16. 50 µl at 2×10^6/ml in IMDM 5% SVF.

Overnight incubation at 37°C.

Then, centrifugation of the plates, removal of 100 µl of supernatants and assaying of IL2 with the commercial kit. Reading at 450 nm.

The values (in pg/ml) are given in the form of a histogram for each sample.

Example 2: In vitro correlation between ADCC and IL-2 release by Jurkat CD16

For this study, 3 anti-D monoclonal antibodies were compared.

The Mab DF5-EBV was produced by human B lymphocytes obtained from a D-negative immunized donor, and immortalized by transformation with EBV. This antibody was used as a negative control given that it was shown to be incapable of eliminating Rhesus-positive red blood cells from the circulation in a clinical trial.

The monoclonal antibody (Mab) DF5-YB2/0 was obtained by expressing the primary sequence of DF5-EBV in the YB2/0 line. The monoclonal antibody R297 and other recombinant antibodies were also expressed in YB2/0.

These antibodies were assayed in vitro for their ability to induce lysis of papain-treated red blood cells using mononuclear cells (PBLs) as effector.

All the assays were carried out in the presence of human immunoglobulins (IVIgs) so as to reconstitute the physiological conditions.

It is thought that IVIgs bind with high affinity to FcgammaRI (CD64). The two Mabs DF5-YB2/0 and R297 induce red blood cell lysis at a level comparable with that of the WinRho antibodies. On the other hand, the Mab DF5-EBV is completely ineffective.

In a second series of experiments, purified NK cells and untreated red blood cells were used as effectors and targets, respectively. After incubation for 5 hours, the anti-D Mabs R297 and DF5-YB2/0 were shown to be capable of causing red blood cell lysis, whereas DF5-EBV remained ineffective.

In these two experiments, the red blood cell lysis was inhibited by the antibody 3G8 directed against FcgammaRIII (CD16).

Taken together, these results demonstrate that the ADCC brought about by the antibody R297 and the antibody DF5-YB2/0 involves FcgammaRIII expressed at the surface of the NK cells.

In the context of the invention, a third series of experiments showed the value of an in vitro assay using Jurkat CD16 cells to evaluate the effectiveness of anti-D antibodies. The antibodies were incubated overnight with Rhesus-positive red blood cells and Jurkat CD16 cells. The release of IL-2 into the supernatant was evaluated by ELISA. A strong correlation between ADCC and activation of the Jurkat cells was observed, which implies that this assay can

be used to discriminate between anti-D antibodies as a function of their reactivity toward FcgammaRIII (CD16).

In conclusion, these data show the importance of post-translational modifications of the structure of the antibodies in terms of their FcgammaRIII-specific ADCC activity. The release of cytokines such as IL-2 reflects this activity.

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